



105

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 1/30, C12N 9/00		A1	(11) International Publication Number: WO 97/34919 (43) International Publication Date: 25 September 1997 (25.09.97)
<p>(21) International Application Number: PCT/DK97/00113</p> <p>(22) International Filing Date: 14 March 1997 (14.03.97)</p> <p>(30) Priority Data: 0305/96 15 March 1996 (15.03.96) DK</p> <p>(71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): RANCKE-MADSEN, Anders [DK/DK]; (DK). LAUSTSEN, Mads, Aage [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK).</p> <p>(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	

(54) Title: METHOD FOR PURIFICATION OF A PROTEIN FROM A PROTEIN CONTAINING SOLUTION

(57) Abstract

A method for purification, and isolation in crystalline form, of a protein from a protein-containing solution comprises: treating the protein-containing solution with a crystallization-effective amount of a water-miscible organic solvent (e.g. a lower aliphatic alcohol or ketone); and isolating the protein in question in crystalline form.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHOD FOR PURIFICATION OF A PROTEIN FROM A PROTEIN-CONTAINING SOLUTION**FIELD OF THE INVENTION**

5

The present invention relates to a simple, inexpensive and very effective method for crystallization of a protein, in particular an enzyme, present in a protein-containing solution, such as a fermentation broth.

10

BACKGROUND OF THE INVENTION

15 Enzymes for industrial purposes are usually provided as liquids or amorphous solids. Their availability as amorphous, rather than crystalline, solids is primarily due to the fact that the known methods for crystallization of enzymes are generally regarded as being too expensive to be used on an industrial scale.

20 There is an abundance of literature concerning crystallization of enzymes. It is difficult to generalize with respect to the outcome of specific crystallization procedures, as the art of enzyme crystallization is highly empirical.

25 Characteristic requirements of most of the hitherto known protein crystallization processes are: pure, concentrated initial solutions; very long crystallization times; and large amounts of chemicals, e.g. salts [see, e.g., Biotechnology and Bioengineering 48 (1995) pp. 316-323].

30

An industrial enzyme crystallization process which employs a water-soluble polymer (such as polyethylene glycol) has been described (see WO 95/01989).

35 SUMMARY OF THE INVENTION

It is an object of the present invention to provide an

effective and inexpensive process by means of which a crystalline protein, in particular an enzyme, may be obtained from a solution containing the protein, and which does not require the addition of large amounts of chemicals such as 5 salts. An important and very valuable feature of the process of the invention is that it permits the isolation of crystalline proteins, particularly enzymes, in a state of high purity, from an impure, protein-containing solution (i.e. a solution which, in addition to the protein in question, contains various other 10 substances), especially a solution which contains other proteins, such as a solution derived from a fermentation broth. The process of the invention makes it possible to obtain high yields using short crystallization times, and is simple, inexpensive, environmentally friendly, and compatible with 15 industrial requirements.

Accordingly, the present invention provides a method for purification, and isolation in crystalline form, of a protein from a protein-containing solution, e.g. a solution which 20 comprises more than one protein, such as a solution derived from a fermentation broth, the method comprising:

- (a) treating the protein-containing solution with a crystallization-effective amount of a water-miscible (water-soluble) organic solvent; and 25
- (b) isolating the protein in question in crystalline form.

It will be understood that protein-containing solutions of 30 particular relevance in the context of the invention are aqueous solutions (i.e., in general, solutions in which water constitutes substantially all of, or at least a major proportion of, the solvent liquid in which the protein of interest is dissolved).

35

DETAILED DISCLOSURE OF THE INVENTION

The present invention thus provides, *inter alia*, a method for crystallizing a protein (or a polypeptide) present in a fermentation broth. In this connection, the term "fermentation" is intended to refer not only to decomposition or 5 transformation processes brought about by microorganisms (such as bacteria or fungi), but also to corresponding processes brought about by the agency of cells of animal or plant origin.

A fermentation broth will, besides the protein or polypeptide 10 of interest, normally contain numerous other substances, such as substrate compounds, e.g. carbohydrates, salts, cells, and other metabolites (such as nucleic acids, and proteins or polypeptides other than the particular protein or polypeptide of interest).

15 When the method of the invention is to be applied to a fermentation broth, it is preferable that the broth is first subjected to one or more solid/liquid separatory techniques, e.g. flocculation, centrifugation, filtration or micro- 20 filtration, or any combination thereof.

The method of the invention appears to work surprisingly well 25 on relatively impure solutions, and it will normally not be necessary to purify a fermentation broth (or a protein-containing solution obtained by, e.g., solid/liquid separatory treatment of a fermentation broth) by means of chromatographic methods (for the purpose, for example, of removing extraneous proteins from the solution) before performing the method of the invention.

30 In another aspect of the invention, the protein-containing solution is concentrated before being treated with the organic solvent(s). Such concentration may suitably be achieved by one or more known procedures, e.g. by ultrafiltration (reverse 35 osmosis) or by evaporation.

In a further aspect of the invention, the protein-containing solution may be subjected to a procedure for removal of low-molecular-weight substances (e.g. salts). Such treatments include diafiltration and dialysis.

5

Whilst concentration of the protein-containing solution is not essential for carrying out the method of the invention, it will often be desirable from the point of view of yield and ease of handling. Generally speaking, the concentration of the protein of interest in the protein-containing solution which is to be treated with organic solvent in accordance with the invention will be in the range of 0.1-25% by weight (% w/w; based on the weight of the protein-containing solution), preferably in the range of 0.5-15% w/w, in particular 5-15% w/w.

10

Preferred proteins in the context of the invention are - as already indicated to some extent above - enzymes, for example:

20 hydrolases (EC 3) [including proteases (peptidases, EC 3.4); carboxylic ester hydrolases (EC 3.1.1), such as lipases (e.g. triacylglycerol lipases, EC 3.1.1.3); glycosidases (EC 3.2), such as amylases (e.g. α -amylases, EC 3.2.1.1, β -amylases, EC 3.2.1.2, and glucoamylases, EC 3.2.1.3), cellulases (e.g. endo-1,4- β -glucanases, EC 3.2.1.4) and xylanases (e.g. xylan endo-25 1,3- β -xylosidases, EC 3.2.1.32)];

30 oxidoreductases (EC 1) [including phenol-oxidases such as laccases (EC 3.10.3.2) and other laccase-related enzymes classified under EC 1.10.3; and peroxidases (EC 1.11), such as those classified under EC 1.11.1.7]; and

isomerases (EC 5) [including xylose isomerases (EC 5.3.1.5)].

35 Enzymes: Enzyme classification numbers (EC numbers) referred to in the present specification with claims are in accordance with the Recommendations (1992) of the Nomenclature Committee of the

International Union of Biochemistry and Molecular Biology,
Academic Press Inc., 1992.

Proteases

5 Proteases (peptidases) which may be crystallized according to the present invention include proteases obtainable by fermentation processes employing cells, such as cells of a microorganism, especially a bacteria or a fungus. Chemically or genetically modified mutants of such proteases are included.

10 The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the Fusarium protease described in WO 89/06270.

20 Suitable commercially available protease enzymes include those sold under the tradenames Alcalase, Savinase, Durazym, and Esperase by Novo Nordisk A/S (Denmark), those sold under the tradenames Maxatase, Maxacal, Maxapem and Properase by Gist-Brocades, those sold under the tradenames Purafect and Purafect OXP by Genencor International, and those sold under the tradenames Opticlean and Optimase by Solvay Enzymes.

25

Lipases

30 Lipases which may be crystallized according to the present invention include lipases obtainable by fermentation processes employing cells, such as cells of a microorganism, especially a bacteria or a fungus. Chemically or genetically modified mutants of such lipases are included

35 Examples of appropriate lipases include a Humicola lanuginosa lipase, e.g. as described in EP 258 068 and EP 305 216, a Rhizomucor miehei lipase, e.g. as described in EP 238 023, a

5 Candida lipase, such as a C. antarctica lipase, e.g. the C. antarctica lipase A or B described in EP 214 761, a Pseudomonas lipase, such as a P. alcaligenes or P. pseudoalcaligenes lipase, e.g. as described in EP 218 272; a P. cepacia lipase, e.g. as described in EP 331 376, a P. stutzeri lipase, e.g., as disclosed in GB 1,372,034, a P. fluorescens lipase, a Bacillus lipase, e.g. a B. subtilis lipase [Dartois et al., Biochimica et Biophysica Acta 1131 (1993), pp. 253-260], a B. stearothermophilus lipase (JP 64/744992) or a B. pumilus lipase (WO 10 91/16422).

15 Numerous cloned lipases may be of interest in the context of the invention. Cloned lipases include the Penicillium camembertii lipase described by Yamaguchi et al. [Gene 103 (1991), pp. 61-67], the Geotrichum candidum lipase [Schimada et al., J. Biochem. 106 (1989), pp. 383-388], and various Rhizopus lipases such as an R. delemar lipase [Hass et al., Gene 109 (1991), pp. 117-113], an R. niveus lipase [Kugimiya et al., Biosci. Biotech. Biochem. 56 (1992), pp. 716-719] and an R. 20 oryzae lipase.

25 Other types of lipolytic enzymes, such as cutinases, may also be crystallized according to the invention. Examples of relevant cutinases are cutinase derived from Pseudomonas mendocina (as described in WO 88/09367), and cutinase derived from Fusarium solani pisi (described, e.g., in WO 90/09446).

30 Especially interesting lipases are commercially available lipases such as M1 LipaseTM, LumafastTM and LipomaxTM (Gist-Brocades), LipolaseTM and Lipolase UltraTM (Novo Nordisk A/S), and Lipase P "Amano" (Amano Pharmaceutical Co. Ltd.).

Amylases

35 Amylases (such as α - or β -amylases), which may be crystallized according to the present invention include amylases obtainable by fermentation processes employing cells, such as cells of a

microorganism, especially a bacteria or a fungus. Chemically or genetically modified mutants of such amylases are included

Relevant amylases include, for example, α -amylases obtained
5 from Bacillus, in particular a special strain of B. licheniformis, described in more detail in British Patent No. 1,296,839. Particularly interesting amylases are commercially available amylases, some non-limiting examples of which are TermamylTM, FungamylTM and BANTM (available from Novo Nordisk
10 A/S), and RapidaseTM and Maxamyl PTM (available from Gist-Brocades).

Related enzymes of interest in the context of the invention include CGTases (cyclodextrin glucanotransferases, EC 2.4.1.19), obtainable from, e.g., bacteria of the genera Bacillus, Thermoanaerobacter or Thermoanaerobacterium.

Cellulases

In the present context, the term "cellulase" refers to an
20 enzyme which catalyses the degradation of cellulose to glucose, cellobiose, cellotriose and other cello-oligosaccharides.

A particularly suitable type of cellulase in the context of the invention is an endo-1,4- β -glucanase (EC 3.2.1.4), preferably a
25 recombinant endo-1,4- β -glucanase.

Cellulases appropriate for crystallization according to the invention include microbial cellulases, notably bacterial or fungal cellulases. Relevant examples of bacterial cellulases
30 are cellulases derived from or producible by bacteria within one of the following genera: Pseudomonas, Bacillus, Cellulomonas, Clostridium, Microspora, Thermotoga and Caldocellum, as well as Actinomycets such as Streptomyces, Termomonospora and Acidothemus. Relevant bacterial species in
35 this connection include Pseudomonas cellulolyticus, Bacillus lautus, Cellulomonas fimi, Microspora bispora, Termomonospora

fusca, Termomonospora cellulolyticum and Acidothermus cellulolyticus.

Relevant fungal cellulases include acid cellulases derived from 5 or producible by fungi within the genera Trichoderma, Myrothecium, Aspergillus, Phanaerochaete, Neurospora, Neocallimastix and Botrytis, in particular a fungus chosen among Trichoderma viride, Trichoderma reesei, Trichoderma longibrachiatum, Myrothecium verrucaria, Aspergillus niger, Aspergillus oryzae, Phanaerochaete chrysosporium, Neurospora crassa, Neocallimastix partriciarum and Botrytis cinerea.

Other interesting fungal cellulases are neutral or alkaline cellulases derived from or producible by fungi within the 15 genera Aspergillus, Penicillium, Myceliophthora, Humicola, Irpepx, Fusarium, Stachybotrys, Scopulariopsis, Chaetomium, Myco-gone, Verticillium, Myrothecium, Papulospora, Gliocladium, Cephalosporium and Acremonium, such as a fungus chosen among Humicola insolens, Fusarium oxysporum, Myceliophthora thermophila, Penicillium janthinellum and Cephalosporium sp., 20 preferably a fungus chosen among Humicola insolens DSM 1800, Fusarium oxysporum DSM 2672, Myceliophthora thermophila CBS 117.65, and Cephalosporium sp. RYM-202.

25 Further cellulases of interest are variants having, as a parent cellulase, a cellulase of fungal or bacterial origin, e.g. a cellulase derivable from a fungal strain within one of the genera Humicola, Trichoderma and Fusarium.

30 Oxidoreductases: Oxidoreductases appropriate for crystallization according to the present invention include peroxidases, and oxidases such as laccases.

Peroxidases

35 Particularly interesting enzymes exhibiting peroxidase activity are those classified under enzyme classification EC 1.11.1.7, as well as fragments of such enzymes exhibiting peroxidase

activity.

5 Peroxidases appropriate for crystallization by the method of the invention are suitably peroxidases producible by microorganisms such as fungi or bacteria. Some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g., Fusarium, Humicola, Trichoderma, Myrothecium, Verticillium, Arthromyces, Caldariomyces, Ulocladium, Embellisia, Cladosporium or Dreschlera, in 10 particular Fusarium oxysporum DSM 2672, Humicola insolens, Trichoderma resii, Myrothecium verrucana IFO 6113, Verticillium alboatrum, Verticillium dahliae, Arthromyces ramosus FERM P-7754, Caldariomyces fumago, Ulocladium chartarum, Embellisia alli or Dreschlera halodes.

15 Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g. Coprinus, Phanerochaete, Coriolus or Trametes, in particular Coprinus cinereus f. microsporus IFO 8371, Coprinus macrorhizus, 20 Phanerochaete chrysosporium (e.g. NA-12) or Trametes (previously called Polyporus), e.g. T. versicolor (e.g. PR4 28-A).

25 Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, e.g. Rhizopus or Mucor, in particular Mucor hiemalis.

30 Some preferred bacteria include strains of the order Actinomycetales, e.g. Streptomyces sphaeroides ATCC 23965, Streptomyces thermophilus IFO 12382 or Streptoverticillium verticillium ssp. verticillium.

35 Other preferred bacteria include Bacillus pumilus ATCC 12905, Bacillus stearothermophilus, Rhodobacter sphaeroides, Rhodomomas palustri, Streptococcus lactis, Pseudomonas purpurascens ATCC 15958 and Pseudomonas fluorescens NRRL B-11.

Further preferred bacteria include strains belonging to

Myxococcus, e.g. M. virescens.

Particularly interesting peroxidases in relation to the method of the invention are recombinantly produced peroxidases, e.g. a peroxidase derived from a Coprinus sp., in particular C. macrorhizus or C. cinereus as described in WO 92/16634, or a variant thereof, e.g. a variant as described in WO 94/12621.

Laccases and Laccase-related enzymes

In the context of the present invention, laccases and laccase-related enzymes include the following: any laccase enzyme comprised by the enzyme classification EC 1.10.3.2; any catechol oxidase enzyme comprised by the enzyme classification EC 1.10.3.1; any bilirubin oxidase enzyme comprised by the enzyme classification EC 1.3.3.5; and any monophenol monooxygenase enzyme comprised by the enzyme classification EC 1.14.18.1.

Appropriate laccases are laccases of microbial origin, notably laccases derived from bacteria or fungi (including filamentous fungi and yeasts). Suitable examples include laccases derivable from strains of Aspergillus, Neurospora, e.g. N. crassa, Podospora, Botrytis, Collybia, Fomes, Lentinus, Pleurotus, Trametes, e.g. T. villosa and T. versicolor, Rhizoctonia, e.g. R. solani, Coprinus, e.g. C. plicatilis and C. cinereus, Psatyrella, Myceliophthora, e.g. M. thermophila, Schytalidium, Polyporus, e.g. P. pinsitus, Phlebia, e.g. P. radita (WO 92/01046), or Coriolus, e.g. C. hirsutus JP 2-238885, in particular laccases obtainable from Trametes, Myceliophthora, Schytalidium or Polyporus.

Organic solvents

Water-miscible organic solvents which are suitable for use in the method of the invention are generally speaking substances which, at standard atmospheric pressure, are liquids at a temperature of, or in the vicinity of, 25°C (including temperatures up to about 30°C). Water-soluble polymers, such as the water-soluble polymers disclosed in WO 95/01989 (some of

which - such as polyethylene glycols at the lower end of the molecular weight range therefor - may be liquids under the above-mentioned temperature and pressure conditions) are not within the scope of water-miscible organic solvents in the 5 context of the present invention.

Suitable water-miscible solvents in the context of the invention include various lower aliphatic alcohols, notably C₁-C₅ aliphatic alcohols, and lower aliphatic ketones, notably C₁-C₅ ketones. 10

Preferred water-miscible organic solvents are solvents which are miscible with water in all proportions. Lower aliphatic alcohols within this category include all of the C₁-C₃ aliphatic 15 alcohols (i.e. methanol, ethanol, 1-propanol and 2-propanol) and the C₄ alcohol tert-butyl alcohol (2-methyl-2-propanol); methanol, ethanol and 2-propanol are particularly preferred. Likewise, a preferred C₃-C₅ ketone is acetone (2-propanone).

20 Other water-miscible, lower aliphatic alcohols such as 2-butanol and isobutyl alcohol (2-methyl-1-propanol), and water-miscible C₃-C₅ ketones such as methyl ethyl ketone (2-butanone) and diethyl ketone (3-pentanone) may be appropriate for use in certain embodiments of the method of the 25 invention.

Further water-miscible organic solvents of interest in the context of the present invention include glycols, notably lower 30 aliphatic glycols (diols), such as ethylene glycol (1,2-dihydroxyethane), 1,2-propylene glycol (1,2-dihydroxypropane) and 1,3-propylene glycol (1,3-dihydroxypropane; also known as trimethylene glycol).

Although the use of water-soluble polymers such as polyethylene 35 glycols and polypropylene glycols for crystallization of industrial enzymes from protein-containing solutions containing various other substances/impurities (including other proteins)

has been described (see WO 95/01989), the present inventors are unaware of any previous disclosure to indicate or imply that simple, water-miscible organic solvents, such as ethanol and the like, could successfully and reliably be applied to obtain crystalline proteins from impure solutions thereof, particularly from solutions containing other protein species (such as solutions derived from fermentation broths). Indeed, such solvents appear to have been rather generally regarded as having a deleterious (e.g. denaturing) effect on protein molecules, such as enzymes, in aqueous solution. Thus, for example, a review article by A. McPherson [Eur. J. Biochem. 189 (1990), pp. 1-23] teaches (see p. 5 therein), in connection with the crystallization of proteins and nucleic acids, that (emphasis added):

15

" . . . common methods for the crystallization of conventional molecules such as evaporation or solvent, dramatic temperature variation, or addition of strong organic solvents are unsuitable and destructive. They must be supplanted with more gentle and restricted techniques."

The latter document reference further teaches (see p. 12
therein), in relation to earlier reports of the use of organic
solvents as precipitants for proteins or nucleic acids, that
25 (emphasis added):

"In general, the most common organic solvents utilized have been ethanol, acetone, butanols and a few other common laboratory reagents. . . It might be noted here that organic solvents have been of more general use for the crystallization of nucleic acids, particularly tRNA and the duplex oligonucleotides. There they have been the primary means for crystal growth. This in part stems from the greater tolerance of polynucleotides to organic solvents and their polyanionic surfaces which appear to be even more sensitive to dielectric effects than are proteins.

The only general rules are that organic solvents should be used at low temperature, at or below 0°C, and they should be added very slowly and with good mixing. . ."

5

The McPherson reference further teaches (see p. 19 therein), in connection with the promotion of crystal growth of proteins, that (emphasis added):

10 "Certainly one major means of promoting periodic bond formation is to ensure that the population of molecules to be crystallized is as homogeneous as possible. . . It means not only that contaminating proteins of unwanted species be eliminated, but that within a target population 15 all individuals assume absolute physical and chemical conformity."

It was thus very surprising to discover that crystallization of a desired protein, in particular an enzyme, from an impure, 20 protein-containing solution (such as a solution derived from a fermentation broth, containing more than one protein species) can be achieved using a simple, water-miscible organic solvent, e.g., ethanol, without a need in general, for example, to operate at low temperatures or to take any special measures in 25 connection with the addition of organic solvent, as taught by McPherson (*loc. cit.*).

The water-miscible solvent employed in the method of the invention will normally be added to the protein-containing solution in question in an amount such that the concentration 30 of solvent in the resulting mixture is in the range of 1-50% by weight (% w/w), often 5-50% w/w, such as 10-50% w/w, e.g. 20-45% w/w. However, in certain cases, such as when 35 crystallizing certain lipases (lipolytic enzymes) by the method of the invention, it may be necessary to employ a much higher percentage of the solvent, e.g. in an amount corresponding to about 90% w/w, or possibly even more, of the solvent/protein-

containing solution mixture.

5 Addition, in accordance with the invention, of water-miscible organic solvent to the protein-containing solution may take place, for example, by: introducing the solvent gradually and essentially continuously; or adding the solvent in several portions of the same or different sizes; or adding all the solvent in one portion. The choice of the manner in which the solvent is added will be based, *inter alia*, on the solubility 10 characteristics not only of the protein or polypeptide of interest, but also of the various substances/impurities other than the protein in question.

15 By way of example, a situation in which a lipase is to be crystallized from a very impure protein-containing solution (derived, e.g., from a fermentation broth containing many components) may be considered: As indicated to some extent above, the solubility of lipases in aqueous media containing a high proportion of, e.g., a lower alcohol such as ethanol is 20 often much greater than that of many other types of enzymes, and crystallization of such a lipase will require a concentration of, e.g., ethanol which is so high that other components of the protein-containing solution will separate from solution (e.g. precipitate) before a solvent concentration 25 which is sufficient to initiate crystallization of the lipase is attained. In such cases, it will be desirable, in order to avoid contamination of the solid, crystalline lipase with solid-phase impurities, to add the organic solvent in portions at intervals sufficient to allow separation, and subsequent 30 removal (e.g. by filtration), of unwanted solid-phase impurities to take place. When such impurities have separated from solution and been removed, further organic solvent may be added to increase the concentration thereof to a level sufficient to initiate crystallization of the enzyme of 35 interest.

If appropriate, more than one water-miscible organic solvent may suitably be employed in the method of the invention. Thus, for example, a crystallization-effective amount of a mixture of two or more water-miscible organic solvents in suitable proportions may be employed to bring about crystallization of the protein of interest. Alternatively, for example, appropriate amounts of different water-miscible organic solvents may be added stepwise to the protein-containing solution.

10

In some embodiments of the method of the invention, appropriate salts may, in addition to water-miscible organic solvent, be added to the protein-containing solution. These will suitably be salts which may be employed in their own right for crystallization of proteins, e.g. enzymes, and such salts include acetates, sulphates ($\text{HSO}_4^-/\text{SO}_4^{2-}$), carbonates ($\text{HCO}_3^-/\text{CO}_3^{2-}$) and phosphates ($\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}/\text{PO}_4^{3-}$), e.g. alkali metal (such as Li^+ , Na^+ or K^+) salts, alkaline-earth metal (such as Ca^{2+}) salts, or Mg^{2+} salts thereof.

15

When, in the context of the invention, such salts are added, together with a water-miscible organic solvent, to a protein-containing solution, the appropriate amount of such a salt to be added will depend, *inter alia*, on the amount of organic solvent which is added. Thus, for example, when performing an embodiment of the method of the invention wherein a solvent concentration (in the mixture of protein-containing solution and water-miscible organic solvent) in the upper end of the normal range (e.g. in the vicinity of 50% w/w), a salt of the type in question will typically be introduced so as to give a salt concentration of up to 0.5-1% w/w. However, if a lower concentration of organic solvent is to be used, it may be appropriate to increase the concentration of the salt.

20

It will be apparent that water-miscible organic solvents of the preferred types will generally be recoverable (e.g. by distillation) from the liquor remaining after crystallization

of the protein in question has been completed, and it will thus be feasible - and from an environmental and economic viewpoint highly desirable - to recycle and re-use such organic solvents in connection with the method of the invention.

5

Adjustment of pH

The pH of the protein-containing solution to which the water-miscible organic solvent(s) is, or has been, added will normally be adjusted to a value which is optimal with respect to 10 crystallization and, preferably, protein stability. The optimum value for a given protein (e.g. enzyme) will depend, *inter alia*, on the exact nature thereof, and may be determined, for example, by performing a trial, typically starting by performing the crystallization procedure at pH 10, then at pH 15 9, pH 8, pH 7.... and so on down to pH 3. If it is found, e.g., that the optimum for a given enzyme is between pH 4 and pH 5, a trial within this pH range may then be made to more precisely establish the optimum pH value. The optimum pH value for most enzymes will normally lie in the range from pH 4 to pH 9.

20

In some cases it may be advantageous to adjust the pH to a value equal to, or in the vicinity of, the isoelectric point (pI) for the protein in question. Thus, in certain embodiments of the method of the invention, pH is adjusted to a value such 25 that $(pI-1) \leq pH \leq (pI+1)$.

In other cases it may be advantageous to alter the pH gradually during the crystallization process (i.e. to employ a pH gradient), or alternatively to carry out stepwise changes in pH 30 during the crystallization process.

Any suitable acid or base may be used to adjust pH. Acids employed may be inorganic or organic. Some examples are hydrochloric acid, sulfuric acid, nitrous acid, phosphoric acid, acetic acid, citric acid and formic acid. Preferred acids are phosphoric acid, formic acid, citric acid, and acetic acid. Preferred bases are sodium hydroxide, potassium hydroxide and

ammonium hydroxide, in particular sodium hydroxide.

Crystallization

Using the method of the invention it will normally be possible
5 to carry out crystallization to a satisfactory extent - without
having to add any seed crystals to start the crystallization -
within a crystallization period of less than 48 hours,
frequently within a period of 36 hours or less, and often
10 within a period of 24 hours or less. Depending upon the
specific conditions applying, including the nature of the
protein or polypeptide to be crystallized and the nature of the
other substances (impurities) present in the protein-containing
solution, crystallization may often be achieved to a
15 satisfactory extent within a period of 12 hours or less, and in
some cases even within a period of about 6 hours.

Temperature

When using the method of the invention, crystallisation of the
protein (e.g. enzyme) of interest will normally take place very
20 satisfactorily at a temperature above 0°C. In general, the
temperature of the protein-containing solution from which the
protein is crystallized will suitably be from 0°C to 40°C,
preferably from 0°C to 30°C, more typically from 5°C to 30°C,
such as from about 7°C to about 28°C.

25 If appropriate, a temperature gradient may be employed, for
example starting at a relatively low temperature (such as a
temperature in the range of from about 0°C to about 7°C) and
then increasing the temperature gradually (or alternatively in
30 steps) up to the final desired temperature over a suitable
period of time (e.g. over a period of up to several hours). In
numerous embodiments of the method of the invention, the final
temperature when employing gradual or stepwise temperature
increase will often be in the vicinity of 25°C (e.g. from about
35 22°C to about 28°C).

Isolation after crystallization

The method of the invention causes the protein, e.g. enzyme, to crystallize. Isolation of the crystalline protein may be accomplished by conventional methods, e.g. by centrifugation and/or filtration, optionally with drying of the isolated 5 product.

If the isolated protein, particularly an enzyme, is subsequently to be granulated, it may be appropriate to carry out a standard granulation procedure directly, starting from 10 the moist, undried product. Drying will then take place during the granulation procedure.

If crystalline products of very high purity are desired, the initial, crystalline product of the method of the invention 15 (which will generally be of rather high purity) may be redissolved to a suitable concentration in an appropriate aqueous medium and recrystallized, e.g. by the method of the invention. Other methods for recrystallization of substantially pure proteins, such as enzymes, may, however be employed. 20 Recrystallization may, of course, be repeated one or more times, as desired.

The final crystalline product may be distributed and/or used as such: If desired, the crystals may, e.g., be dissolved (by the 25 producer or by a consumer) to a suitable concentration in an appropriate medium to give a liquid-phase product.

The present invention further relates to a crystalline protein product obtainable by, or obtained by, a method according to 30 the invention.

The purification/crystallization method of the present invention is believed to be applicable not only to proteins or polypeptides, but also to various other types of substances, 35 including oligopeptides and compounds containing oligopeptide sequences. Such substances include, e.g., certain peptide hormones.

The invention is further illustrated in the following examples, which are not intended to limit the scope of the invention in any way:

5

EXAMPLE 1

Crystallization of *Humicola insolens* cellulase using ethanol

A *H. insolens* cellulase (endo-1,4- β -glucanase) was cloned in 10 *Aspergillus oryzae*. The fermentation broth containing the enzyme in question and other fermentation by-products was subjected to drum-filtration and then ultrafiltration (using Dow DDS Gr61pp membranes; cut-off ca. 20 kD). The ultrafiltration concentrate was further subjected to 15 diafiltration using 2 volumes of deionized water to remove low-molecular-weight substances (such as salts). The resulting solution contained 87 grams of cellulase per liter (constituting 51% w/w of the dry-matter content of the solution), and had a pH of 6.7 and a specific conductivity of 20 0.7 mS/cm.

45.9 g of absolute (99.9%) ethanol was added with stirring to 100 g of the cellulase solution. The temperature was maintained at 27°C. After 17 hours, the resulting crystals were harvested 25 by centrifugation. The yield was 85.4%, based on a determination of cellulolytic activity of the crystalline product redissolved in 0.1% w/w aqueous NaCl solution (eight-fold volume thereof relative to the volume of the crystals).

30 For determination of cellulolytic activity (expressed, e.g., in so-called S-CEVU units), a measured aliquot of the latter enzyme solution (crystalline product redissolved in 0.1% w/w NaCl) is incubated with carboxymethylcellulose (CMC; enzyme substrate). The reaction conditions are as follows:

20

temperature: 40°C (thermostatted)
pH: 7.5 (0.1 M phosphate buffer containing 0.1% w/w PEG 6000)
substrate concentration: 3.11% (in the pH 7.5 buffer)
5 enzyme concentration range: 0.097-0.181 S-CEVU/ml
incubation time: 30 minutes.

10 Cellulolytic degradation of the substrate results in a decrease in viscosity which is measured using a vibration viscosimeter (Mivi 3000, Sofraser, France). The decrease in viscosity is proportional to the cellulolytic activity of the sample. The activity in S-CEVU (Stabilized Cellulase Viscosity Units) is determined relative to an appropriate Novo Nordisk A/S cellulolytic enzyme standard.

15

EXAMPLE 2 (comparative example)

Crystallization of *Humicola insolens* cellulase using PEG 300

20 39.6 g of polyethylene glycol 300 (PEG 300; from BASF) was added with stirring to 100 g of the same cellulase solution as used in Example 1. The temperature was maintained at 27°C. After 20 hours, the resulting crystals were harvested by centrifugation. The yield was 83%, determined as described in Example 1.

25

It is apparent from the above examples that the use of ethanol results in a higher yield of crystalline product than when PEG 300 is employed.

30 **EXAMPLE 3**

Crystallization of *Humicola insolens* cellulase using 2-propanol or acetone

35 A batch of raw fermentation broth containing *H. insolens* cellulase (endo-1,4- β -glucanase) cloned in *Aspergillus oryzae* was diluted with an equal weight of water. After adjustment of

the pH of the diluted broth to 9.5 by addition of 10% (w/w) aqueous sodium hydroxide solution, it was subjected to drum filtration and then to germ-filtration using Seitz EK1 filter pads.

5

The resulting filtrate was subjected to ultrafiltration (using Dow DDS Gr61pp membranes) to a dry-matter content of 22% w/w. The ultrafiltration concentrate was further subjected to diafiltration using 3 volumes of tap water, and then to a 10 carbon treatment using 3% (w/w) of Picatif™ FGV 120 active carbon at pH 5.5 for 2 hours at 30°C. The carbon was then removed by filtration through Seitz K900 filter pads and Seitz EK1 filter pads. The resulting concentrate had a specific conductivity of 1.06 mS/cm.

15

An indication of the level of purity of a given concentrate (cellulase-containing solution) is provided by the magnitude of the ratio of the optical density (OD) of the concentrate at 440 nm to the weight (in grams) of active cellulase in one liter of 20 the solution, i.e. $OD_{440\text{ nm}}/\text{g}$ active cellulase. The higher the value, the lower the purity. For the carbon-treated, filtered concentrate referred to above, the value of this ratio was 21.7, which may be compared with the values given below (vide infra), measured in the same manner, for solutions made up from 25 crystallized cellulase prepared using 2-propanol and acetone, respectively, as the water-miscible organic solvent in the method according to the invention.

After adjusting the pH of the carbon-treated, filtered 30 concentrate to 6.5, aliquots were withdrawn in order to examine the effectiveness of 2-propanol and acetone, respectively, with respect to crystallization of the cellulase of interest.

35 Amounts of 30% w/w and 35% w/w (relative to the weight of the aliquot of concentrate), respectively, of each of the two solvents were added to, and mixed with, respective aliquots of concentrate at a temperature of about 5°C. After 15 minutes at

5 this temperature, the temperature was raised to 28°C and maintained at that temperature for 24 hours. The resulting crystal suspensions were harvested by centrifugation, and each of the crystal cakes were formulated by dissolution in an 8-fold volume of 0.1% (w/w) aqueous sodium chloride solution. The cellulase activity (in S-CEVU; see Example 1) and OD_{440 nm} were measured for each solution. The results are summarized in the table below:

10

Amount of solvent (% w/w)	Yield of active cellulase in formulated product		OD _{440 nm} /(g active cellulase/l) in formulated product	
	2-propanol	acetone	2-propanol	acetone
30	69%	70%	7.7	11.1
35	73%	76%	9.9	11.4

20

25 It is apparent from the above results that high yields and high purities of crystalline enzyme product (in this case a cellulase) are obtainable using 2-propanol or acetone as water-miscible organic solvent in embodiments of the method of the present invention.

CLAIMS

1. A method for purification, and isolation in crystalline form, of a protein from a protein-containing solution comprising more than one protein, the method comprising:
 - (a) treating said protein-containing solution with a crystallization-effective amount of a water-miscible organic solvent; and
 - (b) isolating the protein in question in crystalline form, with the proviso that said water-miscible organic solvent is not a water-soluble polymer.
2. The method according to claim 1, wherein said protein-containing solution is derived from a fermentation broth.
3. The method according to claim 2, wherein said protein-containing solution is a solution obtained by subjecting said fermentation broth to a solid/liquid separation procedure.
4. The method according to any one of claims 1-3, wherein said protein-containing solution is concentrated prior to addition of said organic solvent.
5. The method according to any one of claims 1-4, wherein the concentration of said protein in said protein-containing solution, prior to addition of said organic solvent, is in the range of 0.1-25% w/w, preferably in the range of 0.5-15% w/w, such as in the range of 5-15% w/w.
6. The method according to any one of the preceding claims, wherein said crystallization takes place at a temperature above 0°C.
7. The method according to any one of the preceding claims,

wherein the protein to be purified is an enzyme.

8. The method according to claim 7, wherein said enzyme is selected from the group consisting of proteases, lipases, cellulases, amylases and oxidoreductases.

9. The method according to any one of the preceding claims, wherein said water-miscible organic solvent is selected from the group consisting of methanol, ethanol, 2-propanol and acetone.

10. A crystalline protein product obtainable by a method according to any one of claims 1-9.

INTERNATIONAL SEARCH REPORT

1

International application No.

PCT/DK 97/00113

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/30, C12N 9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, PAJ, MEDLINE, DERWENT BIOTECH ABSTRACTS, BIOSIS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Eur. J. Biochem., Volume 189, 1990, Alexander McPherson, "Current approaches to macromolecular crystallization", page 1 - page 23, the whole document --	1-10
X	WO 9110677 A1 (BASF AKTIENGESELLSCHAFT), 25 July 1991 (25.07.91), table 1 --	1-10
A	WO 9501989 A1 (NOVO NORDISK A/S), 19 January 1995 (19.01.95) -- -----	1-10

 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

1 July 1997

Date of mailing of the international search report

12- 07- 1997

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Patrick Andersson
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/06/97

International application No.

PCT/DK 97/00113

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9110677 A1	25/07/91	AT 111481 T CA 2067790 A DE 4001238 A DE 59007173 D EP 0511221 A,B SE 0511221 T3 JP 5503511 T US 5304310 A	15/09/94 19/07/91 25/07/91 00/00/00 04/11/92 10/06/93 19/04/94
WO 9501989 A1	19/01/95	AU 7121494 A BR 9407047 A CN 1126477 A EP 0707594 A FI 960073 A JP 8512294 T	06/02/95 13/08/96 10/07/96 24/04/96 08/01/96 24/12/96